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Examination of the Mitochondrial Health of the Basigin Null Mouse Retina

Kristine Anne V. Pablo
University of North Florida

Examination of the mitochondrial health of the Basigin null mouse retina

By

Kristine Anne V. Pablo

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CERTIFICATE OF APPROVAL

The thesis "Examination of the mitochondrial health in the Basigin null mouse retina," submitted by Kristine Anne V. Pablo

Approved by the thesis committee: Date

Signature Deleted

11.28.11

Judith D. Ochrietor
Committee Chair Person

Signature Deleted

11.29.11

Dr. Michael Lentz

Signature Deleted

11.30.11

Dr. Cliff Ross

Accepted for the Department of Biology:

Signature Deleted

12-1-11

Dr. Courtney Hackney
Chairperson

Accepted for the College of Arts & Sciences:

Signature Deleted

12-1-11

Dr. Barbara Hetrick
Dean

Accepted for the University:

Signature Deleted

12-20-11

Dr. Len Roberson
Dean of the Graduate School

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Abstract

Basigin gene products are cell adhesion molecules that are expressed by photoreceptor cells, Müller cells and endothelial cells of the mammalian retina. Previous studies have suggested that a lactate shuttle exists between the photoreceptor cells and the Müller cells, with Basigin being an essential component in this shuttle. Deletion of the Basigin gene in mice results in blindness with an eventual retinal degeneration. It was hypothesized that the lactate shuttle between photoreceptors and Müller cells does not form in Basigin null mice and that the blindness is attributed to faulty photoreceptor metabolism. Therefore, the purpose of this study was to determine whether the mitochondria of the Basigin null mice are metabolically active in the absence of the lactate shuttle. Mitochondrial health in the Basigin null mouse retina was assessed by a variety of assays, including ELISA analyses to measure Cytochrome c concentration and expression of autophagy-specific proteins. Mitotracker dyes were used to stain the mitochondria of Basigin null and normal retinas to determine the number of metabolically active mitochondria and the total number of mitochondria. The results showed that apoptosis and autophagy are not occurring in the Basigin null animals at a rate greater than that of the normal animals. The Mitotracker assay showed that there is a ~60% decrease in the total number of mitochondria in the null animals compared to their normal counterparts. A recalculation of the Cytochrome c assay in light of the reduced number of mitochondria in Basigin null mice revealed that apoptosis is likely occurring in these animals prior to the first signs of cytoarchitectural changes in the tissue. These results suggest that in the absence of the lactate shuttle in the Basigin null animals, the photoreceptors are unable to perform oxidative phosphorylation at the necessary rate, thus decreasing the number of mitochondria, which results in limited photoreceptor functionality, hence blindness.

Chapter 1: Introduction

OVERVIEW OF THE EYE

The eye is a robust optical instrument composed of two positive lenses that produce real images on the retina, initializing the visual process (Artal, 2008). The mammalian retina lies loosely embedded in fatty tissue, each in its bony orbit. It is externally covered by a resistant and flexible tissue called the sclera except in the anterior region – which is covered by the cornea, allowing light to pass through the eye (Artal, 2008). Light reaching the eye is first transmitted and refracted by the cornea and passes through an anterior chamber filled with aqueous humor. Posterior to this chamber is the iris, a muscle that controls the pupil size, which in turn limits the amount of light passing through the eye. The crystalline lens lies posterior to the iris and allows images to pass through the posterior chamber filled with vitreous humor and be focused on the retina (Artal, 2008). A diagram of the mammalian eye is shown in Figure 1.1 .

THE NEURAL RETINA

The retina is a thin neural tissue (~ 0.25 mm) composed of ten layers and lines the back of the eye (Figure 1.2). It contains light-sensitive photoreceptor cells (rods and cones) and four classes of neurons – horizontal, bipolar, amacrine and ganglion cells – along with astrocytes and Müller glial cells (Dowling, 1987). The Müller cell extends from the ganglion cell layer to the outer limiting membrane (OLM) and is closely associated with the photoreceptors. These neurons are organized into three nuclear layers separated by two synaptic plexiform layers, where all retinal synapses are found (Dowling, 1987). The pigmented epithelium is the most posterior layer of the retina and is responsible for the phagocytosis of the ends of rods and cones to maintain optimum function. Old discs containing visual pigment molecules in the outer

Figure 1.1. Horizontal section through the mammalian eye

The eye is made of three coats enclosing three transparent structures. The outermost layer is composed of the cornea and the sclera. The middle layer consists of the choroid, ciliary body and the iris- a muscle that controls the size of the pupil and the amount of light that enters the eye. The inner layer is the retina, which gets its circulation from the vessels of the choroid (Barton et. al, 2007).

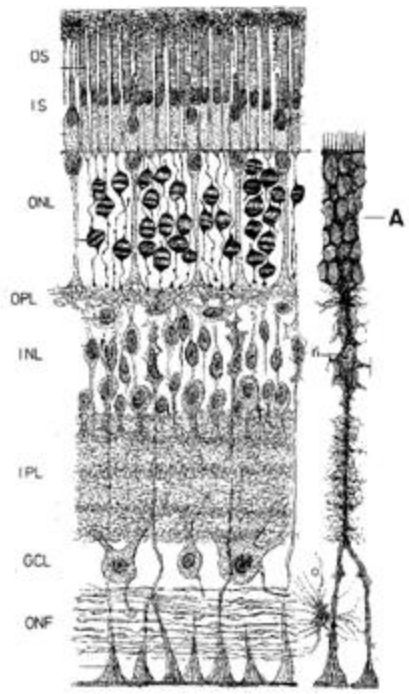

Figure 1.2. Section of the mammalian retina as drawn by Ramon Y Cajal in 1893

The ganglion cell layer (GCL) houses the cell bodies of the ganglion cells. Bipolar cell and ganglion cell layer synapses are located in the inner plexiform layer (IPL), whereas the cell bodies of the bipolar cells and horizontal cells are found within the inner nuclear layer (INL). The synapses between photoreceptors (rods and cones) and second order neurons (bipolar cells and horizontal cells) are found within the outer plexiform layer, while the nuclei of the photoreceptors are found within the outer nuclear layer (ONL). The bacillary layer contains the inner (IS) and outer segments (OS) of the photoreceptors. The Müller cell (A) closely associates with photoreceptors and extends from the outer limiting membrane to the nerve fiber layer. Müller cell bodies are located in the INL (Andrews, 1999).

segment (OS) are constantly engulfed by the apical process of the pigmented epithelium and new discs are added at the base of the OS cilium in a diurnal cycle (Young, 1971). Phagocytosis by the pigmented epithelium is an integral element in establishing a balanced rate of OS growth, renewal, and disposal (Spira, 2005). The choroid lies between the pigmented epithelium and the sclera. It is a brown, pigmented coat that consists of blood vessels and connective tissue that serves to nourish the eye.

The light travels through all the layers of the retina to the outer segments, where the photons of light activate retinal, and a cascade of signaling events hyperpolarizes the cells. In rods, photons captured in the OS trigger the conversion of rhodopsin into an active form (Meta II rhodopsin; Simos,2001). The Meta II rhodopsin initiates a series of events that lead to the hydrolysis and deactivation of cyclic guanosine monophosphate (cGMP) by the enzyme phosphodiesterase (Simos,2001). The low levels of cGMP close the cationic channels, causing the cell membrane to hyperpolarize. A similar mechanism occurs in cones. Photoreceptors signal to the second order neurons (bipolar cells, horizontal cells, amacrine cells) and the signal converges on the ganglion cells, whose axons become the optic nerve. The signal is sent through the optic nerve bundle, ending in the lateral geniculate nuclei in the brain (Dowling, 1987). The photoreceptor membrane can return to its depolarized state when the photoexcited form of rhodopsin returns to its photosensitive state or there is a reduced level of intracellular Ca^{2+}, leading to an enzymatic reactivation of cGMP (Simos,2001) .

Vision begins in the retina when rod and cone photoreceptor cells detect light from visual images and transduce them into neural signals (Ribelayga, 2008). Rods and cones primarily function under different lighting conditions: rods mediate dim light (scotopic) vision at night and cones mediate bright light (photopic) and color vision during the day (Ribelayga, 2008). The

opsin proteins used dictate the wavelengths of light detected in rods and cones. A mature mammalian retina contains about 7 million cones and 75 to 150 million rods. In mice, the photoreceptors are mostly rods, and cones comprise less than 3% of the total photoreceptors (Sharma, 2003). These photoreceptors also differ in structure, as seen in Figure 1.3. Measurements from isolated and intact retinas show that the photoreceptors, particularly the inner segments, have high oxygen consumption (Hoang, 2002) which indicates that aerobic respiration occurs at a fast rate. This is important because ATP is necessary for neurotransmitter release from photoreceptor neurons.

MITOCHONDRIA

Active neurons need ATP to support synthesis, release and recycling of neurotransmitter molecules, movement of materials to and from the cell body by axoplasmic transport, maintenance of normal resting potential, and the recovery of action potential through the sodium and potassium pump. The ATP generated in the mitochondria must be expended to maintain normal cytoplasmic ion concentrations (Martini, 1999). The mitochondria are membrane enclosed organelles found in most eukaryotic cells, ranging from 0.5-10 μm in diameter (Henze, 2003). This organelle is composed of compartments which carry out specific functions, including the outer membrane, the inter-membrane space, the inner membrane, and the matrix. In addition to ATP generation, the mitochondria are involved in cell signaling, cell differentiation, and cell growth and death.

Mitochondria in adult mammalian photoreceptors are sequestered to the inner segments and the axon terminals of the OPL (Stone, 2008; Figure 1.3). The photoreceptor layer lacks intrinsic blood vessels and is supplied oxygen by diffusion from the choroid. Mitochondria

O'Brien 1982 and Young 1970

Figure 1.3. Rod and Cone Photoreceptors

Vision begins in the retina when rod and cone photoreceptor cells detect visual images and transduce them into neural signals (Ribelayga, 2008). Rods and cones primarily function under different lighting conditions: rods mediate dim light (scotopic) vision at night and cones mediate bright light (photopic) and color vision during the day (Ribelayga, 2008). The mitochondria are found in the inner segment (Stone, 2008).

migrate to these areas where oxygen concentration is high (Stone, 2008). Photoreceptors are metabolically active cells which need large amounts of ATP.

The electron transport chain (ETC) in the inner membrane of mitochondria allows the ATP synthase to use the flow of electrons to generate ATP (adenosine triphosphate) from ADP (adenosine diphosphate) and inorganic phosphate (Karp, 2008). The ETC is comprised of several complexes that participate in oxidation-reduction reactions. Complex I (NADH coenzyme Q Reductase) accepts electrons from NADH and passes them to coenzyme Q, which also receives electrons from Complex II (succinate dehydrogenase). Coenzyme Q passes electrons to Complex III (cytochrome b-c complex) which passes electrons to Cytochrome c. This complex in turn passes electrons to Complex IV (Cytochrome c oxidase) which uses the electron gradient to reduce molecular oxygen to water (Karp, 2008). Energy from electron transfer allows Complexes I, III and IV to pump protons to the intermembrane space. Protons then flow down the concentration gradient through the ATP synthase to the matrix. Protons passing through the synthase power the process of ATP synthesis.

APOPTOSIS

In addition to the metabolic function of the mitochondria, this organelle also functions in the regulation of apoptosis (Hoang, 2002). Apoptosis is a form of programmed cell death important in the development of tissues and homeostasis of multicellular organisms. During apoptosis, cells die in a highly coordinated manner, with characteristic structural factors including membrane blebbing, reduction in cell volume, and chromatin condensation (Bossy-Wetzel, 2000; Figure 1.4)

Figure 1.4. Characteristics of an apoptotic and necrotic cells

(1), The disruption of plasma membrane and organelles is observed in necrotic cells. (2), An apoptotic (A) and a normal (N) cell are shown; note the characteristic chromatin rearrangement in A. (3) A necrotic cell is shown. Numerous lesions appear on the cell surface. (4). Surface blebbing is evident on the apoptotic cell. (5) The regular distribution of nuclear pores is visible. (6) The nuclear envelope shows a characteristic apoptotic clustering (asterisk) of nuclear pores. (Robinson, 2006)

Mitochondrial Cytochrome c, which functions as an electron carrier in the respiratory chain, translocates into the cytosol in cells undergoing apoptosis. Cytochrome c is a water-soluble protein with a net positive charge and is normally loosely associated with the mitochondrial intermembrane space (Bossy-Wetzel, 2000). This protein functions in the respiratory chain by interacting with redox (reduction-oxidation) partners of complex III and IV. During apoptosis, Cytochrome c translocates into the cytosol from the mitochondria and binds with apoptotic protease factor -1 (Apaf-1), ATP, and procaspase 9 to create a complex called an apoptosome. The apoptosome cleaves procaspase 9 to its active form – caspase 9 – which then activates the effector caspase 3. Caspase 3 is responsible for chromatin condensation and DNA fragmentation (Bossy-Wetzel, 2000). Cytochrome c release from the mitochondria is considered an early event in the apoptotic process (Bossy-Wetzel, 2000).

AUTOPHAGY

Autophagy is a tightly regulated pathway involving the lysosomal degradation of cytoplasmic organelles or components. Studies have noted the connection between autophagy and mitochondrial turnover. Mitochondrial quality control is the process whereby mitochondria undergo successive amounts of fusion and fission with a dynamic exchange of components to segregate functional components from damaged components (Gottlieb, 2010). During autophagy, a double membrane vesicle called an autophagosome sequesters organelles for delivery to the lysosome. The sequestered contents are degraded in the lysosome, allowing cells to eliminate damaged components through catabolism and maintain nutrient and energy homeostasis (Kroemer, 2008). When mitochondria are selectively degraded, the process is known as mitophagy.

Several proteins are involved in the autophagic process. The molecule named Autophagy 1 (APG1) stimulates autophagy, leading to autophagy-dependent restriction of cell growth and ultimately high levels of apoptotic activity. It is also a negative regulator of mTOR (mammalian target of rapamycin) signaling (Scott, 1997). The molecule named mTOR is a protein that regulates vital cell growth processes (Chen, 2009). Beclin 1 (BECN) plays a role in two fundamentally important cellular pathways: autophagy and apoptosis. It is thought to function as an autophagy protein as part of a complex with a Class III PI3 kinase (Phosphoinositide-3), and Vps34, which regulates endolytic processes that occur during late stage autophagy (Scott, 1997). The molecules APG16L and APG5L are expressed during autophagosome formation and the GTPase RAB 24 is expressed during the formation and regulation of autophagic vacuoles (Scott, 1997). The APG5L and APG16L complex is essential for the elongation of autophagic isolation membranes. This complex (APG5L and APG16L) initially associates in uniform distribution with small vesicle membranes (Scott, 1997).

Although autophagy is a natural process that removes damaged organelles, it may also be stimulated by multiple forms of cellular stress, including nutrient deprivation, insufficient growth factors, hypoxia, reactive oxygen species, DNA damage, and pathogens (Kroemer, 2008). Nutrient stress is the most potent inducer of autophagy and is known to start the pathway within minutes of starvation. In mice, within 24-48 hours of starvation, most cells in most tissues displayed an increased number of autophagic proteins (Kroemer, 2008). A diagram of the autophagic process is shown in Figure 1.5.

Autophagy is a major degradative pathway for organelles and long lived proteins and is essential for the survival of neurons. Previous studies implicated defective autophagy in the pathogenesis of Alzheimer's disease, particularly the massive buildup of incompletely digested

Figure 1.5. Autophagic pathway

Several proteins are involved in the autophagic process. The molecule named APG (autophagy) 1 stimulates autophagy, leading to autophagy-dependent restriction of cell growth and ultimately high levels of apoptotic activity. It is also a negative regulator of mTOR (mammalian target of rapamycin) signaling (Scott, 1997). The molecule named mTOR is a protein that regulates vital cell growth processes (Chen, 2009) BECN (Beclin 1) plays a role in two fundamentally important cellular pathways: autophagy and apoptosis. It is thought to function as an autophagy protein as part of a complex with a Class III PI3 kinase, Vps34, which regulates endolytic processes that occur during late stage autophagy (Scott,1997). The molecules APG16L and APG5L are expressed during autophagosome formation and the GTPase RAB 24 is expressed during the formation and regulation of autophagic vacuoles (Scott, 1997). The APG5 and APG16L complex is essential for the elongation of autophagic isolation membranes. This complex initially associates in uniform distribution with small vesicle membranes (Scott, 1997).

substrate within the axon and dendrites (Nixon, 2011). Other neurodegenerative diseases, including Huntington's disease and Parkinson's disease, may be caused by the failure of autophagosomes to recognize their correct cargo, incorrectly sequestering proteins (Martinez-Vicente et al., 2010).

MONOCARBOXYLATE TRANSPORTERS

The isolated mammalian retina is known for its large production of lactate, both in the presence and absence of oxygen. This conclusion is bolstered by *in vitro* studies of isolated retinas, which release large quantities of lactate into the medium in response to light (Gerhart, 1999). Cellular respiration includes the conversion of glucose to pyruvate via glycolysis and subsequent Krebs cycle and oxidative phosphorylation to produce energy stored in the form of ATP (Su, 2009). The production of lactate when glycolysis outpaces the remaining aerobic process enables the electron acceptor NAD^+ to be regenerated (Poitry-Yamate, 1995). Highly glycolytic cells, like Müller cells of the retina and skeletal muscle fibers can release lactate and a proton to prevent acidification of the cell interior (Poitry-Yamate, 1995).

Monocarboxylates are charged species that cannot pass the plasma membrane by free diffusion but require a proton linked monocarboxylate transporter (MCT). These MCT transporters catalyze facilitated diffusion of lactate with a proton. Fourteen MCT members have been identified (Wilson et al., 2009), with characteristic shared motifs (Halestrap and Price , 1999). For instance, MCT2 has ~60 % amino acid sequence identity to MCT 1, while MCTs 5-7 have ~ 30% amino acid sequence identity to MCT 1.

Figure 1.6. Schematic of MCT 1 within the plasma membrane

MCT 1 is made of twelve transmembrane helices with a long loop between transmembranes 6 and 7, and both the carboxy- (C-) and amino- (N-) termini facing the cytosol (Halestrap and Price, 1999). Cuff and Shirazi-Beechey (2005) determined the site of transcription initiation for the MCT 1 gene (3410 bp) and demonstrated that its gene structure to comprise of five exons interrupted by four introns.

The MCT 1 protein consists of twelve transmembrane helices with a long loop between transmembranes 6 and 7, and both the carboxy- (C-) and amino- (N-) termini facing the cytosol (Halestrap and Price, 1999; Figure 1.6). Transport is proton-linked by a symport mechanism that affects cellular concentration of both monocarboxylates and hydrogen ions (Gerhart, 1999). In addition

to MCT 1, MCT isoforms 2-4 also catalyze the transport of monocarboxylates such as L-lactate across the plasma membrane. MCT 8 is a high affinity thyroid hormone transporter, whereas MCT 10 (TAT1) is an aromatic amino acid transporter. The other members of the MCT family remain to be characterized (Wilson et al., 2009)

The MCT 1 protein is detected in four retinal cell types including pigmented epithelial cells, photoreceptor cells, Müller cells, and blood vessel endothelial cells (Gerhart, 1999; Philp et al., 2003). The occurrence of MCT 1 in the Müller cell plasma membrane is consistent with the proposed role of these cells as net producers and transporters of lactate (Gerhart, 1999). Furthermore, studies have shown that there is a high concentration of lactic acid and low pH near the Müller cell microvilli that extends into the subretinal space and the outer nuclear membrane (Gerhart, 1999). MCT 1 in the inner segments of photoreceptors, the region of the cell that contains mitochondria, also functions to move lactate across the membrane and into the cell.

BASIGIN

Basigin was first described by Linser in 1986 as the 5A11 antigen and was originally thought to be Müller cell specific (Linser et al., 1986). It has since been localized to the plasma membrane of the retinal pigment epithelium (RPE), photoreceptor cells and their inner segments, and blood vessel endothelial cells of the retina, in addition to the Müller cells (Ochrietor et al, 2001). This protein has been subsequently identified in various tissues and cell lines, and it is

known as CE9 in rats (Finneman et al., 1997), 5A11 or HT7 in chicken (Seulberger et al., 1990) and extracellular matrix metalloproteinase inducer (EMMPRIN) or leukocyte activation antigen in humans (Biswas et al , 1995). These proteins, with different names, are identical in form.

The Basigin gene is located on chromosome 10 in mice and the protein is synthesized by a secretion system including the rough endoplasmic reticulum (rER) and the Golgi apparatus (Ikagura et al., 1998). The gene consists of eight exons spanning 7.5 kb of DNA (Miyauchi et al., 1995; Ochrietor et al., 2003). The Basigin protein is 27 kDa that becomes a 50 kDa integral membrane glycoprotein belonging to the immunoglobulin (Ig) superfamily. The protein possesses two Ig domains, a hydrophobic transmembrane domain and a short cytoplasmic tail (Figure 1.7). The first immunoglobulin-like domain of the Basigin molecule is encoded by the second and third exons and the second immunoglobulin-like domain by the fourth and fifth exons. The fifth exon encodes not only the C proximal portion of the second immunoglobulin-like domain, but also the transmembrane domain and a small portion of the cytoplasmic domain (Miyauchi et al., 1995).

Studies done by Ochrietor et al.(2003) identified a second form of Basigin, named Basigin-2. Basigin-2 is a transcript of ~1.8 kb and is the result of the splicing of an exon into the Basigin transcript (Ochrietor et al., 2003). The Basigin-2 protein has a similar structure to the Basigin protein with an additional Ig domain in the extracellular portion composed of 116 amino acids (Ochrietor et al., 2003; Figure 1.7). Basigin-2 is found only in the retina, specifically the surface of photoreceptor cells and their inner segments (Ochrietor et al., 2003).

In terms of function, Basigin is a cell-adhesion molecule known to be involved in a variety of cell and tissue processes. As reviewed by Ochrietor and Linser (2004), Basigin plays a

Figure 1.7. Basigin gene products

Basigin and Basigin-2 are members of the immunoglobulin superfamily (IGSF) and have Ig-like structures. Basigin is a 50 kDa integral plasma membrane glycoprotein belonging to the immunoglobulin (Ig) superfamily and possess two Ig domains, a hydrophobic transmembrane domain and a short cytoplasmic tail. Basigin-2 has a similar structure to Basigin with an additional Ig domain (open circles) in the extracellular portion composed of 116 amino acids, making the protein 55 kDa (Ochrietor et al., 2003).

role in metalloproteinase induction required for wound healing and tumor progression. Basigin has also been implicated in mammalian oocyte maturation, spermatogenesis, blastocyst implantation, as well as thymic development and HIV infection (Ikagura et al.,1998; Renno et al., 2002; Pushkarsky et al., 2001).

More than a decade ago, a strain of mice was generated in which the gene for Basigin was deleted by molecular means (Ikagura et al., 1998). Basigin null animals have several deficiencies. For example, Basigin null mice exhibit infertility and disruptions in fertilization (Ikagura et al, 1998). Approximately 96% of Basigin null embryos are lost around the time of uterine implantation (Ikagura et al, 1998; Kuno et al, 1998). In addition, these animals exhibited lower sensitivity to irritating odors (Ikagura et al., 1998), have a reduced response to foot shock (Naruhashi et al., 1997) and are blind (Hori et al., 2000; Ochrietor et al., 2002).

Studies have shown that Basigin and Basigin-2 are both absent in Basigin null mice (Ochrietor et al., 2003). The null animals are visually impaired at the time of eye opening (2 weeks of age) despite normal retina architecture at that age (Ochrietor et al., 2002). At visual maturity (3 weeks of age), the photoreceptor outer segments appear less dense and shorter than those of control animals, and at 8 weeks, retinal degeneration is first observed (Ochrietor et al., 2001). Degeneration of the photoreceptors continues such that by 8 months of age, the entire photoreceptor layer is missing (Hori et al., 2000; Ochrietor et al., 2001).

Philp et al., (2003) demonstrated that Basigin is necessary for the targeting of MCTs to the plasma membrane and is essential for normal retinal function. A model showing the interaction of MCT 1 and Basigin is shown in Figure 1.8. When Basigin is absent, MCT 1 expression is not observed at the plasma membrane, but rather observed in intracellular vesicles (Philp et al., 2003). MCT 1 is the primary lactate carrier found in the Müller cells and inner

Figure 1.8. A model of the Basigin and MCT 1 complex (Kirk et al., 2000)

Basigin is shown as the black molecule, whereas MCT 1 is shown as the gray molecule. Basigin exists as a dimer within the membrane and each polypeptide also associates with MCT 1. The transmembrane domain of Basigin interacts with MCT 1 via hydrophobic interactions (Finch et al., 2009).

segments of the retinal photoreceptors. It was hypothesized that a lactate shuttle exists in the retina. It is thought that the Basigin gene products adhere Müller cells to photoreceptors so that MCT 1 can efficiently transfer lactate from the Müller cells to the photoreceptors which then use lactate as an energy source (Ochrietor et al., 2009). In the absence of MCT 1, lactate from Müller cell metabolism is no longer available for energy metabolism in the photoreceptors, which could lead to the blindness phenotype (Philp et al., 2003).

Since lactate is not being transported out to the photoreceptors in Basigin null mice, there is a possible accumulation of this monocarboxylate in the Müller cells. In a study done by Su et al (2009), it was determined that high concentrations of lactate reduces the extracellular pH in tumor cells. The purpose of this present study was to determine whether mitochondrial metabolism or overall health is compromised in Basigin null photoreceptors. It has been hypothesized that the mitochondria in Basigin null mice photoreceptors are less active than those of littermate controls due to the lack of lactate presentation to those cells.

Chapter 2: Materials and Methods

Generation of mouse retina cell extracts

Basigin null mice and heterozygous littermate controls were sacrificed according to an accepted protocol (UNF IACUC #10-009) and the eyes were immediately removed. Neural retinas were isolated from individual eye cups in Phosphate-buffered Saline (PBS; 50 mM potassium phosphate, 150 nM NaCl, pH 7.2) in a Petri dish. The tissue was transferred to a 1.5 mL centrifuge tube (USA Scientific, Ocala, FL) and centrifuged in an Eppendorf 5415 Centrifuge for 1 minute at 16,100 ×g. The residual PBS was removed and discarded with a fine tip transfer pipette (SAMCO Scientific, San Fernando , CA). The pelleted tissue was lysed using 200 µl of Cell extraction solution (10 mM Tris, pH 7.4, 100 mM NaCl, 1mM EDTA, 1mM EGTA, 1mM NaF, 20 mM $Na_4P_2O_7$, 2mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate; Invitrogen Corporation, Carlsbad, CA) containing 1mM phenylmethanesulfonylfluoride (PMSF; Sigma-Aldrich, St. Louis, MO) and protease inhibitor cocktail (Pierce/Thermo Scientific, Rockford, IL) . The tissue was incubated on ice for 30 minutes with vortex mixing every 10 minutes, followed by centrifugation in an Eppendorf Centrifuge 5415 at 13,000 rpm for 10 minutes at 4°C. Cleared lysates (supernatants) were transferred to clean .5 mL centrifuge tubes using fine tip transfer pipettes. The protein concentration was determined via a protein assay.

Protein assay of mouse retina cell extracts

Bovine Serum Albumin (BSA; Pierce/Thermo Scientific) standards were prepared in 1.5 mL centrifuge tubes, as described in Table 2.1. Each of the mixtures was vortex mixed and 5 µl of each mixture was transferred to duplicate wells of a 96 well plate (Corning Incorporated, Corning, NY). Samples (2.5 µl) of mouse retina cell extracts were added to 2.5 µl of deionized water, vortex mixed, and transferred to other wells of the same 96 well plate. Coomassie (Bradford) stain (250 µl; Pierce/Thermo Scientific) was added to the wells containing the BSA standards and the mouse retina samples. The absorbance of the solution in each well of the plate was measured at 595 nm using a Bio-Tek platereader (Winooski, VT) and a standard curve for BSA was generated using Microsoft Excel software (Redmond, WA). The concentrations of the mouse retina cell extracts were calculated from the equation of the standard curve.

Cytochrome c assay

Cytochrome c standard (10 ng; Invitrogen Corporation, Carlsbad, CA) was reconstituted with 2 mL of the Standard Diluent Buffer (Invitrogen Corporation) and allowed to incubate for 10 minutes at room temperature. Standard Diluent Buffer (0.25 mL) was added to six 1.5 mL centrifuge tubes, and 1:2 serial dilutions of the standard were made as described in Table 2.2. Solutions were vortex mixed between dilutions.

The Cytochrome c standards generated were transferred to wells commercially coated with an antibody to Cytochrome c (Invitrogen Corporation). The normal and Basigin null mouse retina cell extracts were diluted 1:10 in Standard Diluent Buffer (100 µl total volume) in 1.5 mL centrifuge tubes and then transferred to other commercially coated wells.

Table 2.1. BSA Standard Preparation

Tube #	BSA (µl)	Deionized water(µl)	BSA concentration (mg/mL)
1	0	20	0
2	2	18	0.2
3	4	16	0.4
4	6	14	0.6
5	8	12	0.8
6	10	10	1.0
7	12	8	1.2
8	14	6	1.4
9	16	4	1.6
10	18	2	1.8
11	20	0	2.0

Table 2.2. Dilution of Cytochrome c Standard

Concentration	**Standard**	**Diluent Buffer**
5 mg/mL	0.25 ml of 5 mg/mL Standard	0 mL
2.5 ng/mL	0.25 mL of the 5 mg/mL standard	0.25 mL
1.25 ng/mL	0.25 mL of the 2.5 ng/mL standard	0.25 mL
0.625 ng/mL	0.25 mL of the 1.25 ng/mL standard	0.25 mL
0.312 ng/mL	0.25 mL of the 0.625 ng/mL standard	0.25 mL
0.156 ng/mL	0.25 mL of the 0.312 ng/mL standard	0.25 mL
0.078 ng/mL	0.25 mL of the 0.156 ng/mL standard	0.25 mL
0 ng/mL	0 mL	0.25 mL

Wells were covered with Parafilm (Pechiney Plastic Packaging, Menasha, WI) and incubated at room temperature for 2 hours. Excess solutions were aspirated from the wells with a fine tipped transfer pipette and discarded. A working wash solution was prepared in a 50 mL Corning tube (Corning Incorporated) using 1 mL of the 25x wash Buffer concentrate (Invitrogen Corporation) diluted in 24 mL of deionized water. Wells were washed four times each with 0.4 mL of the working wash solution for 15-30 seconds each. The wash buffer was removed with a fine tipped transfer pipette. Next, each well received 100 µl of the anti-Cytochrome c (Biotin Conjugate), except for the chromogen blank. The wells were covered with Parafilm and incubated for one hour at room temperature. The solution was removed from each well with a fine tipped transfer pipette and the wells were washed four times with 0.4 mL of the working wash solution for 15-30 seconds. The Streptavidin -horseradish peroxidase (HRP) working solution was prepared by diluting the Streptavidin HRP (Invitrogen Corporation) 1:100 in HRP diluent buffer (Invitrogen Corporation). The Streptavidin HRP working solution (100 µl) was added to each well except the chromogen blanks. Wells were covered with Parafilm and incubated for 30 minutes at room temperature. The solution was removed from each well with a fine tipped transfer pipette and the wells were washed four times as previously described. Stabilized chromogen (100 µl) was added to all of the wells and incubated for 30 minutes at room temperature. Stop solution (10 µl; Invitrogen Corporation) was added to all the wells and the absorbance was measured at 450 nm using a Bio-Tek platereader.

A standard curve for Cytochrome c was generated using Microsoft Excel software so that the Cytochrome c concentrations for the normal and Basigin null mouse retina cell extracts and controls could be determined.

Autophagy assay

Mouse retina cell extracts from normal and Basigin null animals were diluted to 100 ug/mL in PBS in a 1.5 mL centrifuge tube and transferred to wells (100 µl to each) of a 96-well plate (Corning Incorporated). Wells designated as the spectrophotometric blanks received 100 µl of PBS. The plate was covered with Parafilm and incubated for two days at 4°C. The protein samples and PBS were removed from respective wells with a transfer pipette. Primary antibodies specific for RAB 24, APG1, APG16L, BECN1 and APG5L (Abgent Antibodies, San Diego, CA) were each diluted 1:100 in PBS in a 1.5 mL centrifuge tube and transferred (100 µl each) to appropriate wells of the assay plate. The wells were covered with Parafilm and incubated for 30 minutes at 37°C. The solutions were removed from the wells with fine tipped transfer pipettes and the wells were washed three times with 0.3 ml of PBST (PBS containing 0.05% Tween 20). Alkaline phosphatase-conjugated goat-anti-rabbit secondary antibody (Pierce/Thermo Scientific) was diluted 1:1000 in PBS in a 1.5 mL centrifuge tube and 100 µl was added to each appropriate well. The wells were covered with Parafilm and incubated for 30 minutes at 37°C. The solution was removed to Alkaline phosphatase Substrate (PNPP; Pierce/Thermo Scientific; 100 µl) and was added to all the wells and the plate was incubated for approximately 30 minutes at room temperature. The reactions were stopped by adding 50 µl of 2N NaOH to each well. The absorbance of the solutions in each well was measured using a Bio-Tek platereader at 405 nm. The absorbance measured was directly correlated to the relative concentration of the target protein present the samples. All runs were performed in duplicate. Microsoft Excel software was used to generate the graphical representation of the data.

Mitotracker Assay

Mitotracker Red FM and Green FM (Invitrogen Corporation) dyes were diluted in DMSO to a working concentration of 1mM and incubated for 30 minutes at 37°C.

Basigin null mice and heterozygous littermate controls were sacrificed at post natal day 18 (P18) according to an accepted protocol and the eyes were immediately removed. Neural retinas were isolated from individual eye cups in PBS and then transferred to 1.5 mL centrifuge tubes containing both Mitotracker Red and Mitotracker Green, each diluted 1:1000 in PBS (500 µl total volume). The tissues were disrupted by passing through a 1-200 µl volume micropipette tip and incubated for 2 hours at 37°C. The tissues were pelleted by centrifugation in an Eppendorf 5415D Centrifuge at 13,000 rpm for 10 minutes. The supernatant was removed with a fine tipped transfer pipette and the tissue was resuspended in 200 µl of 37^{o}C PBS. The solution was mixed by vortexing and 100 µl of each solution was transferred to a black Nunc 96 well plate (Thermo Scientific, Rochester, NY). The absorbance was measured using a Biotek Fluorescence FLx800 plate reader at 550-650 nm for Mitotracker Red and 400 nm for Mitotracker Green.

Microsoft Excel Software was used to determine the relative percentages of active mitochondria in each sample and generate a graph of the results.

Chapter 3: Results

The Basigin null mouse is characterized as possessing several phenotypic abnormalities. Both male and female null mice are sterile, as Basigin is necessary for spermatogenesis and embryo implantation into the endometrial layer of the uterus (Igakura et al., 1998). Basigin null mice do not run from offensive odors like their control littermates (Ikagura et al., 1998), they have a high threshold for pain (Naruhashi et al., 1997), and they are blind from the time of eye opening (Hori et al., 2000; Ochrietor et al., 2002). It has been proposed that the blindness observed in these animals stems from faulty metabolism in the retina in the absence of Basigin gene product expression in that tissue (Philp et al., 2003).

It has been proposed that a lactate shuttle exists within the retina, in which Basigin gene products interact with MCTs 1 and 4 to deliver lactate from the Müller cells to the photoreceptor cells. Lactate is a monocarboxylate that serves as a source of pyruvate for metabolism in vertebrate nervous tissues, which is subsequently converted to acetyl CoA and fuels oxidative phosphorylation (Lewandowski et al., 1995). Basigin null mice do not express MCT 1 and MCT4 on the plasma membranes of Müller cells or photoreceptor cells (Philp et al., 2003). It has therefore been proposed that in the absence of the lactate shuttle complex, in the Basigin null mouse retina, the photoreceptor cells are not sufficiently nourished by the Müller glial cells and they never function (Ochrietor and Linser, 2004). Since mitochondria are the organelles responsible for cellular respiration, the aim of this project was to assess mitochondrial health in the Basigin null mouse retina.

Assay of Cytochrome c

Initially, mitochondrial health was assessed via a Cytochrome c assay. Basigin null and littermate control retinas were harvested at three weeks and three months of age and protein lysates were isolated. The assay uses an ELISA format to compare Cytochrome c concentration in samples relative to a standard curve of Cytochrome c at known concentrations at the ages of 3 and 12 weeks. It was determined that the concentration of Cytochrome c was reduced in the Basigin null mouse retinas compared to the concentrations observed in retinas of littermate controls (Figure 3.1).

Assay of autophagy induction

The results of the initial assay, in which it was determined that Cytochrome c concentrations were lower in Basigin null mice compared to littermate controls, suggested that mitochondria and Cytochrome c may be sequestered via mitophagy within the Basigin null mouse retinas. Mitophagy is a specific form of autophagy, which is a tightly regulated pathway involving the lysosomal degradation of mitochondria (Yue at al., 2003). Although it is a natural process that removes damaged organelles, autophagy is also stimulated by multiple forms of cellular stress, including nutrient deprivation, insufficient growth factors, hypoxia, reactive oxygen species, DNA damage, and pathogens (Kroemer, 2008). Therefore, it is plausible to suggest that autophagy occurs in the Basigin null mouse retina. The same normal and Basigin null mouse retina lysates (post-natal day [P] 18) generated for the Cytochrome c analyses were subjected to ELISA analyses using antibodies specific for proteins known to be expressed within the autophagy pathway. The proteins assayed are expressed at various points of autophagosome

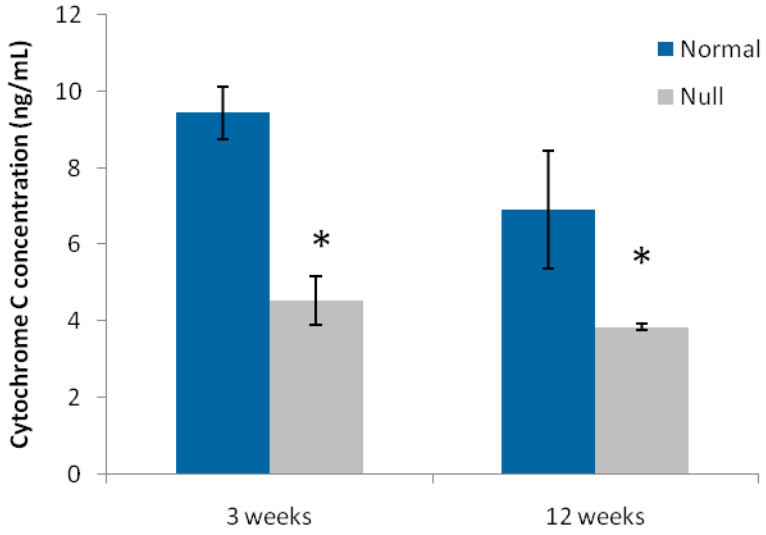

Figure 3.1. Cytochrome c assay

Cytochrome c concentrations (ng/mL) in Basigin normal and null mice retinas were determined using a Cytochrome c assay (Invitrogen Corporation). Samples of normal and Basigin null mouse retina lysates were compared to a standard curve of known Cytochrome c concentrations to determine the concentration of Cytochrome c within each sample. Runs were performed in duplicate. The average concentration at each age is plotted and the error bars indicate the standard deviation. Normal animals are indicated by the blue bars, whereas the Basigin null animals are indicated by the gray bars. * $p < 0.05$ via Student t-Test (comparison of normal to null at each age)

formation. Specifically, the assay measured relative amounts of APG1, which stimulates autophagy; APG16L and APG5L , which are expressed during autophagic vesicle formation; BECN1, which regulates endolytic processes during late stage autophagy; and the GTPase RAB24, which is expressed during the formation and regulation of autophagic vacuoles (Scott, 1997). As shown in Figure 3.2, no significant increase in expression of any autophagy proteins was observed in the Basigin null mice as compared to the littermate controls. Indeed, the expression levels of autophagy proteins APG16L, RAB24 and BECN1 were actually reduced when compared to the normal animals.

Assay of mitochondrial activity

Since the first two assays gave perplexing results, the next step was to directly determine the relative activities of mitochondria in normal and Basigin null mice using Mitotracker dyes (Invitrogen Corporation). Retinas from postnatal day 12 Basigin null mice and littermate controls were isolated and mechanically dissociated in a saline buffer. The retinas were then incubated with a mixture of Mitotracker Red, which stains metabolically active mitochondria, and Mitotracker Green, which stains all mitochondria. The red fluorescence measured at 650 nm provided a relative measure of mitochondria activity, since the dye is only taken up by mitochondria that possess a membrane potential. The green fluorescence measured at 510 nm allowed the total numbers of mitochondria to be normalized between the normal and null samples. It was determined that the percentages of active mitochondria are similar between the normal and Basigin null samples, as shown in Figure 3.3A. However, the percentages of total mitochondria are reduced by ~50% in the Basigin null mice when compared to those of control animals (Figure 3.3B).

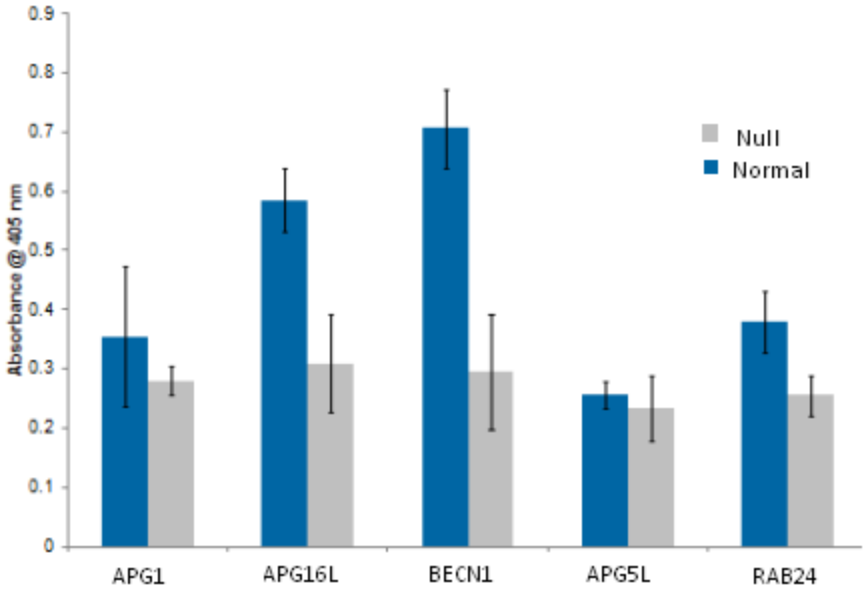

Figure 3.2. **Relative expression of autophagy proteins in normal and Basigin null mouse retinas**

Samples of normal and Basigin null mouse retina protein lysates were compared for the relative expression of autophagy proteins via ELISA analysis. Runs were performed in duplicate and the average absorbance at 405 nm was plotted. Error bars indicate the standard deviation for each sample. Normal mice are indicated by the blue bars, whereas Basigin null mice are indicated by the gray bars.

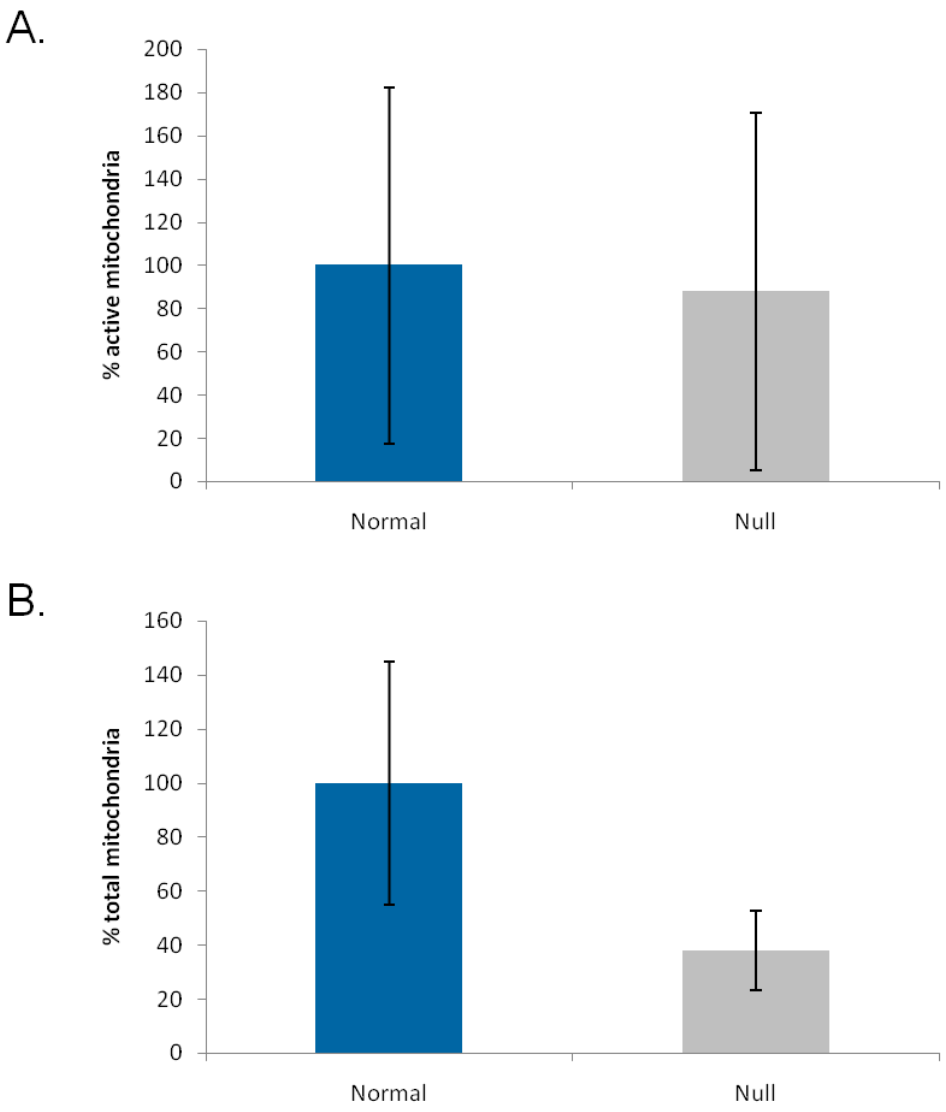

Figure 3.3. Mitotracker analyses of mitochondria from normal and Basigin null mouse retinas

The percentage of active mitochondria (A) were measured using Mitotracker Red FM dye (Invitrogen Corporation) and the percentage of total mitochondria (B) were measured using Mitotracker Green FM dye (Invitrogen Corporation). Runs were performed in triplicates. The value obtained for normal mouse retinas was set to 100% and those for Basigin null mouse retinas were compared. The error bars represent the standard deviations. Normal mice are indicated by the blue bars, whereas Basigin null mice are indicated by the gray bars.

To assess mitochondrial activity and numbers during postnatal development of the mouse retina, an additional assay was carried out. Retinas from normal mice at postnatal days 1, 7, 14, 21 and 28 were isolated and mechanically dissociated in a saline buffer. The retinas were then incubated with a mixture of Mitotracker Green and Mitotracker Red, as described. Figure 3.4 shows the results of the study. It was determined that the number of active mitochondria within a mouse retina is greatest at visual maturity (postnatal day 21).

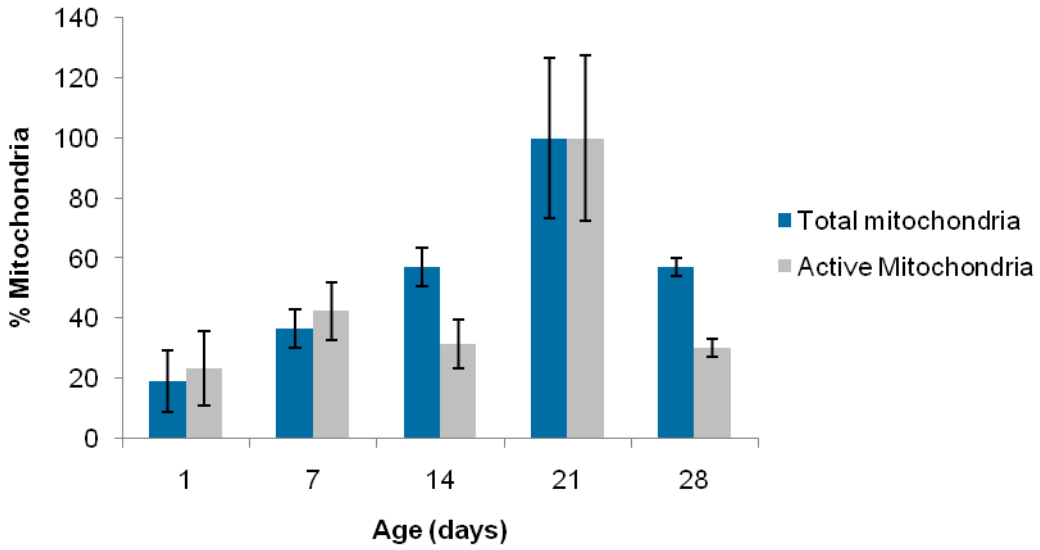

Figure 3.4. Mitotracker assay of mouse retina

Mitotracker Green was used to determine the concentration of the total number of mitochondria (blue) while Mitotracker red was used to determine the concentration of active mitochondria (gray). Runs were performed in triplicate. Absorbance was adjusted to reflect percentages of concentrations relative to P21 (retinal visual maturity). The error bars represent the standard deviations.

Chapter 4: Discussion

Basigin gene products are found on the surface of Müller cells, photoreceptor cell bodies and a portion of the inner segment of the retina (Ochrietor et al., 2001). It was reported that the retinas of Basigin null mice undergo retinal degeneration preceded by the retardation of photoreceptor maturation, and the blindness phenotype in these animals (Ochrietor et al., 2001). The purpose of this study was to determine the possible cause of blindness in the Basigin null mice by examining the mitochondrial health of the retina. It was hypothesized that the lack of a lactate shuttle in the Basigin null mouse retina causes the inactivity of the photoreceptors, leading to blindness.

Lactate has been shown to constitute an adequate energy substrate for skeletal muscles, testis, and various nervous tissues (Pellerin, 2003). Poitry-Yamate et al., (1995) proposed that the net production and release of lactate by Müller cells serves to fuel mitochondrial oxidative metabolism and glutamate resynthesis in photoreceptors. The spatial relationship of Müller cells and the photoreceptors makes the trafficking of metabolites possible (Poitry-Yamate, 1995), likely mediated by MCT 1 and Basigin (Philp et al., 2003).

Initially, Cytochrome c and autophagy assays were performed to assess mitochondrial health in the Basigin null mouse retina. Mitochondrial Cytochrome c is a water soluble protein that functions as an electron carrier in the respiratory chain. The release of Cytochrome c from the mitochondria – the organelle that regulates apoptosis- precedes caspase activation and is considered an early event in the apoptotic process (Finucane et al., 1999). These characteristics make the detection of this protein an effective tool in determining whether apoptosis is occurring

in a tissue. In this study , it was used to determine whether apoptosis in Basigin null mouse retinas could be detected prior to photoreceptor degeneration . Using ELISA, it was shown that there is a significant decrease in the concentration of Cytochrome c in the Basigin null mice compared to normal counterparts. These data suggest that apoptosis is not upregulated in the Basigin null mouse retina, as compared to its littermate controls.

Autophagy, like apoptosis, is a natural and tightly regulated pathway, but involves the lysosomal degradation of whole cytoplasmic organelles and their components. Several neurodegenerative diseases are caused by defective autophagy, including Alzheimer's disease, in which a massive buildup of undigested substrate is seen within the axons and dendrites (Nixon, 2011). Failure of autophagosomes to recognize their correct cargo can lead to incorrect sequestration of proteins which in turn leads to Huntington's and Parkinson's disease (Martinez-Vicente et al., 2010). Autophagy can be triggered by multiple forms of cellular stress including nutrient deprivation (Kroemer, 2008). It was proposed, based on the low levels of Cytochrome c in the null mouse retinas, as compared to the controls, that mitophagy, a specialized form of autophagy in which whole mitochondria and their components are destroyed may occur in the Basigin null mouse retina. Using ELISA, it was shown that there was not a significant increase in the levels of autophagy proteins in the null animals compared to their normal counterparts. These data suggest that autophagy is not upregulated in the Basigin null mouse retina.

Mitotracker dyes (red and green) were used to directly detect the mitochondrial metabolic activity in the Basigin null and normal retinas. Mitotracker Green is a fluorescent mitochondrial dye that localizes to the mitochondria regardless of mitochondrial membrane potential. Mitotracker Red is a fluorescent dye that stains mitochondria in live cells and its accumulation depends on the presence of membrane potential (Invitrogen Corporation). Dissociated retinas

were stained with both Mitotracker Red and Green so that mitochondrial activity (Mitotracker Red) could be compared using samples that have been normalized for total number of mitochondria (Mitotracker Green). While it was determined that there was no significant difference in the percent of active mitochondria between the two groups, it was revealed that Basigin null mice have a ~ 50% less mitochondria in their retinas than age matched controls. This is a significant finding, since the ages tested are before retinal degeneration begins in these animals (Ochrietor et al., 2001), meaning that the two groups should have comparable numbers of photoreceptors. This finding also explains the results of the Cytochrome c assay, as lower numbers of mitochondria will yield a lower concentration of Cytochrome c.

It is of interest to note that mitochondrial numbers increase during post natal eye development and peak at retinal maturity (P21 , Figure 3.4) . The numbers of mitochondria then decrease by half at the adult age tested. The majority of mitochondria present are active at all ages tested, except P14 and P28, in which only ~ 50% of mitochondria are active. Since mitochondria turnover approximately every ten days (Alberts, 1994), it is likely that newly "born" mitochondria that are not yet active were detected at those ages.

Although the Mitotracker assay indicates a reduction in total numbers of mitochondria in Basigin null mouse retinas, it is not known which cells of the retina have low numbers of mitochondria. A recent study in which Basigin expression within a human malignant melanoma cell line was knocked down indicated that glycolytic output diminishes in the absence of Basigin (Su et al., 2009). Similar to what occurs in the Basigin null mouse retina, Basigin downregulation in the cell line led to downregulation of the expression of MCT 1 at the plasma membrane. Lactate produced by the Basigin knockdown melanoma cells cannot be exported from the cells and feedback inhibition reduces the rate of glycolysis (Su et al., 2009). It is

therefore likely that Müller cells of the Basigin null retina undergo a similar phenomenon. Glycolysis rates would not likely affect total numbers of mitochondria within the same cell. It is therefore plausible to propose, based on studies by Poitry-Yamate (1995), that the reduction in mitochondrial numbers is attributed to the photoreceptors.

In conclusion, a model for the metabolic uncoupling that is proposed to occur in the Basigin null mouse retina is shown in Figure 4.1. Müller cells and photoreceptors are in close association with each other. Blindness in the Basigin null mice is thought to be attributed to the lack of a lactate shuttle between these cells (Philp et al., 2003). In the Basigin null Müller cells, lactate is being produced and is not transported to the photoreceptors because of the absence of MCT 1 and MCT 4 on the plasma membrane, which may lead to the acidification of the cell and decrease in the glycolysis rate. The Basigin null photoreceptors are not likely receiving lactate for oxidative phosphorylation, leading to the decrease in the number of mitochondria, as seen in the Mitotracker assay. Mitochondrial numbers are dictated by the metabolic activity of the cells or tissues (Alberts, 1994). Since little lactate –mediated metabolism is thought to occur in Basigin null photoreceptors, it is likely that these cells have reduced numbers of mitochondria. Less mitochondria means less cellular energy and therefore depressed synaptic activity. As synaptic activity decreases, neuronal degeneration occurs. It is clear that this examination of the mitochondrial health in the Basigin null retina proved to be essential in understanding the mechanism of retinal dystrophy and degeneration seen in these animals.

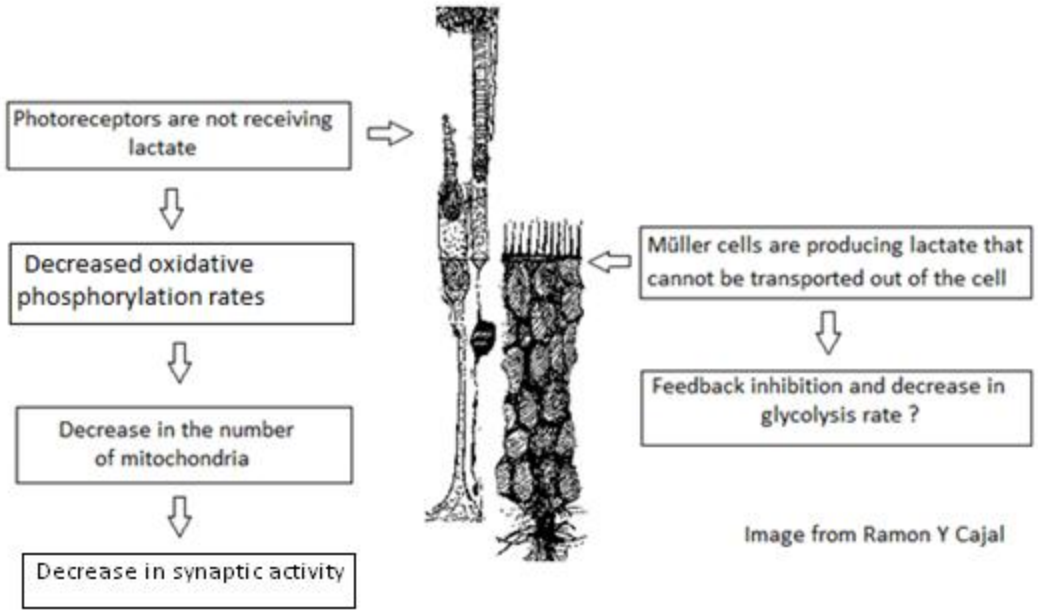

Figure 4.1. Metabolic uncoupling in the Basigin null mouse retina

Lactate is not delivered from Muller cells to photoreceptors, which results in decreased oxidative phosphorylation and subsequent decreases in mitochondria. The lack of energy in photoreceptors may account for depressed ERGs in Basigin null mice, and hence their blindness from the time of eye opening. Neurons that do not work well die, therefore, degeneration of the photoreceptors is eventually observed. Lactate buildup in Muller cells leads to acidification of those cells and likely decreased glycolytic activity.

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VITA

Name of author

- Kristine Anne V. Pablo

Date and place of birth

-

Degrees awarded

- B.S, Biology, University of North Florida, May 2007, *Cum Laude*

Honors and awards

- Graduate Scholars Program Grant, Fall 2009
- Graduate Scholars Program Grant, Spring 2010
- SENN/GASCNC Travel Grant Award, Spring 2010

Professional Experience

- Graduate Teaching Assistant, University of North Florida, January 2009- May 2011

Presentations

- 2010 South East Nerve Net (SENN) and Georgia/South Carolina Neuroscience Consortium (GASCNC) conference. Atlanta, Georgia, USA, March 05 - 07, 2010